Investigating the role of NF-Y transcription factors in the response to abiotic stresses between Vitis vinifera and Vitis amurensis

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Research Article

Keywords: NF-Y TFs family, Salt stress, Drought stress, Antioxidant enzyme activity

Posted Date: April 12th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4154844/v1

Abstract

Nuclear factor Y transcription factors (TFs) play a crucial role in the response of plants to abiotic stresses. However, there is a lack of research on the comparative analysis of evolutionary relationship, real-time quantitative fluorescence PCR (RT-qPCR), and TFs functions of *NF-Y* TFs between *Vitis vinifera* (*V. vinifera*) and *Vitis amurensis* (*V. amurensis*). In this study, a total of 27 and 26 *NF-Y* TFs were identified in *V. vinifera* and *V. amurensis*, respectively, and were divided into 3 subgroups. Subcellular localization prediction revealed that the *NF-Ys* TFs were mainly located in the nucleus. Interestingly, the conserved five motif analysis showed that the NF-YB protein sequences were more conserved, whereas the amino acid sequences of NF-YA and NF-YC showed varying degrees of loss and gain in both species. Thus, these sequences may be closely related to the functions performed by grapevine. RT-qPCR analysis of 'Pinot Noir' and 'Zuoyouhong' plantlets demonstrated that the expression levels of *VaNF-YA6*, *VaNF-YB5*, *VvNF-YA3*, *VvNF-YA5*, and *VvNF-YC2* were significantly upregulated under 400 mmol·L^{-1} NaCl and 10% PEG treatments. Consistently, subcellular localization showed that the *VaNF-YA6-GFP* fusion protein was functioned primarily in the nucleus. Overexpression of *VaNF-YA6* in *Arabidopsis thaliana* (*Arabidopsis*) can significantly enhance the tolerance to salt and drought stresses by activating antioxidant enzyme activities in *Arabidopsis*.

Key Message

A total of 27 and 26 NF-Y TFs were identified between V. vinifera and V. amurensis. VaNF-YA6 positively regulates the salt and drought tolerance in transgenic Arabidopsis.

Introduction

NF-Y TFs, known as the CCAAT binding factor (*CBF*) the heme activator factor (*HAP*), is widely expressed in eukaryotes. It can specifically recognize and bind CCAAT elements, and regulate the transcription of downstream genes (Myers & Holt 2018; Kahle et al. 2005; Quach et al. 2015). However, the NF-Y TFs only encode 1–2 proteins in animals and fungus (Qu et al. 2021). Previous studies found that the *NF-Y* TFs have three subunits, including NF-YA (CBF-B or HAP2), NF-YB (CBF-A or HAP3), and NF-YC (CBF-C or HAP5) (Kim et al. 1996; Li et al. 2016). They can bind at specific sites to form heterotrimeric complexes that play an important roles in plant growth and development (Sinha et al. 1995; Laloum et al. 2013).

Previous studies have confirmed that the inner regions of NF-YA conserved domains contain A1 and A2 regions, which had 20 amino acids and 21 amino acids, respectively (Calvenzani et al. 2012; Frontini et al. 2004; Maity and de Crombrugghe 1992; Xing et al. 1994; Zhao et al. 2009). A1 is mainly responsible for binding to the NF-YB and NF-YC subunits, while A2 is mainly combined with CCAAT elements to regulate related responsive genes in the nucleus (Antonio Leyva-Gonzalez, et al. 2012). Meanwhile, the NF-YB subunit is smaller than the NF-YA subunit in the nucleus and cytoplasm, and contains a conserved central structural region, which consists of three α-helixes and two β-turns, and is involved in binding to DNA and interacting with other proteins (Sinha et al. 1996; Yamamoto et al. 2009). Similarly, NF-YC protein is mainly found in the cytoplasm and nucleus, with a molecular weight between that of NF-YA and NF-YB also contain a HFM domain, which also is also composed of three α-helixs and two β-turns. The HFM domain is also a crucial region for binding to DNA and interacting with other proteins (Malviya et al. 2016). Additionally, NF-YC proteins also had an αC conserved domain containing an α-helix of 7-amino acids (Malviya et al. 2016). In the NF-YA/B/C complex formation process, the NF-YB and NF-YC subunits associate in the cytoplasm to form the NF-YB/C complex, which then associates with NF-YA subunits in the nucleus to form a heterotrimer (Cao et al. 2011).

NF-Y TFs have been successfully identified in various species, including *Arabidopsis*, *Oryza sativa* (rice), *Triticum aestivum* (wheat), *Ziziphus jujuba* Mill. (wild jujuba), *Vitis vinifera* (*V. vinifera*), *Solanum tuberosum* (potato), etc. (Panzade et al. 2022; Ren et al. 2016; Siefers et al. 2009; Stephenson et al. 2007; Thirumurugan et al. 2008; Xuanyuan et al. 2022; Zhang et al. 2021). The earliest reported showed that members of the NF-Y TF family play important roles from flower bud differentiation to fruit growth and development, mainly including hypocotyl elongation and growth, root system elongation

and thickening, photomorphological growth of plants, flowering regulation process, embryo formation process, endosperm development, and other processes (Antonio Leyva-Gonzalez et al. 2012; Du et al. 2022; Mu et al. 2013; Niu et al. 2020; Zhang et al. 2021; Zhang et al. 2022). In *Arabidopsis*, *AtNF-YB9*, also known as *LEC1*, is the first reported TF found to play a critical role and influence in the formation and development of embryos and seed maturation (Kwong et al. 2003; Lee et al. 2003). Likewise, *NF-YB6* (*LEC1-Like*) plays a significant role in the embryo formation process (Junker et al. 2012). Overexpression of *AtNF-YB2* promotes the proliferation and expansion of cells in the root tip region of plants, which is beneficial for the absorption of water and mineral uptake (Ballif et al. 2011). Additionally, studies have shown that *AtNF-Y* TFs also play a crucial regulatory role in the flowering stage of *Arabidopsis* (Hou et al. 2014). In rice, *OsNF-YB11* expression is downregulated during flowering to control the plant photoperiod and induce flowering (Wei et al. 2010). In the absence of *OsHAP3A*, the chlorophyll content of plants decreased significantly (Miyoshi et al. 2003). In the castor oil plant, *RcNF-YB2* and *RcNF-YB12* play important roles in seed formation, development, substance accumulation and storage (Wang et al. 2018). *TaNF-YA-B1* and *TaNF-YB3* in wheat also positively regulate in the root growth and development (Qu et al. 2015; Stephenson et al. 2010). Moreover, a total of three *NF-YA* and two *NF-YB* TFs have been identified as potential regulators of the ripening process in tomato fruit using virus-induced gene silencing (VIGS) and meticulous examination of subcellular localization (Li et al. 2016).

Previous research has shown that the NF-Y TF family also has a crucial and indispensable function in diverse stress conditions (Dai et al. 2021; Panzade et al. 2022; Wang et al. 2022). In *Arabidopsis*, *AtNF-YA5* plays a key role in drought resistance. Overexpression of *AtNF-YA5* showed the significant tolerance to drought conditions, while mutant strains knocking out *AtNF-YA5* showed higher susceptibility to drought-induced stress (Li et al. 2008). In rice, overexpression of the *OsNF-YA2* can confer enhanced salt and drought tolerance (Alam et al. 2015). This effect is also observed in transgenic plants overexpressing the drought-responsive *AtNF-YB1*, as well as in the maize homolog *ZmNF-YB2*, which shows increased resistance to drought stress (Nelson et al. 2007). Furthermore, several other TFs, namely *GmNF-YA3*, *CdNF-YC1*, and *ZmNF-YA14*, are involved in mechanisms that confer drought resistance (Li et al. 2008). Meanwhile, *GmNF-YA* has the ability to regulate the activity of genes involved in the high-salt response, thereby enhancing the resistance of maize to salt stress (Lu et al. 2021). Overexpression of *GmNF-YA10* in *Arabidopsis* improves the drought resistance of *Arabidopsis* by increasing survival rate, water content, abscisic acid, proline, and chlorophyll content (Yu et al. 2020). Similarly, overexpressing of soybean *GmNF-YC14* of the in *Arabidopsis* also showed good drought resistance (Gusmaroli et al. 2001). In transgenic maize, *ZmNF-YB2* led to an improvement in photosynthetic rate and chlorophyll content under drought stress conditions (Yu et al. 2021). Additional results consistent with the above have also been found in wheat (Stephenson et al. 2011).

Grapes are one of the world's largest cultivated and highest-yielding fruit trees, with considerable economic and medicinal value. However, salinity, alkalinity and drought stress have a significant impact on grape production and quality. Therefore, it is crucial to explore genes related to grape stress response, to improve grape stress resistance and to increase grape yield. Despite the submission of numerous *NF-Y* TFs from *V. vinifera* and *V. amurensis* to the grape genome database, there is a lack of studies that have carried out a comparative analysis of their evolutionary relationships, RT-qPCR, and TFs functions. Therefore, the aim of this scientific investigation was to identify and analyze the *NF-Y* TFs between *V. vinifera* and *V. amurensis*. The paper comprehensively addresses these issues using various techniques such as phylogenetic trees, homologous relationships, gene structure analysis, identification of motifs and codon usage, *cis*-acting elements, and RT-qPCR. In addition, the functional validity of *VaNF-YA6* was specifically assessed under conditions of salt and drought stress. Consequently, the results of the study contribute to a more comprehensive understanding of the evolutionary relationship and gene function of *NF-Y* TFs.

Materials and Methods

1.1 Plant materials and condition

In the study, plantlets of ‘Pinot Noir’ and ‘Zuoyouhong’ were used as *V. vinifera* and *V. amurensis* for RT-qPCR of *VvNF-Y* and *VaNF-Y* TFs, respectively. All materials were grown on 50 mL GS solid medium supplemented with 6 $g \cdot L^{-1}$ agar, 30 $g \cdot L^{-1}$ sucrose, and 0.2 $mg \cdot L^{-1}$ indole-3-acetic acid (IAA), and then subcultured for 30 d. Materials with consistent growth were selected for further treatment with 50 mL GS liquid medium supplemented with 400 $mmol \cdot L^{-1}$ NaCl and 10% PEG, and the equal amount of distilled water was used as the control. Then, the leaves of ‘Pinot Noir’ and ‘Zuoyouhong’ were collected after 24 h and frozen at -80 ℃ for RNA extraction. Meanwhile, transgenic *Arabidopsis* was screened on 1/2 MS solid media (pH = 5.7) with 30 $mg \cdot L^{-1}$ kanamycin, and positive lines were transferred to plastic pots filled with vermiculite and soil (1:3). Four-week-old *Nicotiana benthamiana* (*N. benthamiana*) were used for observation of subcellular localization. All materials were kept in a growth chamber at 22/25 ℃ under an 8 h dark/12 h light cycle with 10000 lx of white light.

1.2 Identification of NF-Y TFs

The protein sequences of the *NF-Y* TFs in *Arabidopsis* and rice were downloaded from Phytozome v12.1: Home (https://phytozome.jgi.doe.gov/). These sequences were then used as queries to perform Blastp in the National Center for Biotechnology Information (NCBI). The genome, CDS, and protein sequences of the *NF-Y* TFs of *V. vinifera* were downloaded. Additionally, TBtools was used to extract the genome, CDS, and sequences of *NF-Y* TFs in *V. amurensis*, then the all NF-Y protein sequences of *V. vinifera* and *V. amurensis* were identified specific domain (CBFB_NFYA, PF02045; CBFD_NFYB_HMF, PF00808; HAP5 super family, cl30738) using HMME (https://www.ebi.ac.uk/Tools/hmmer/) and NCBI, respectively.

1.3 Physical and chemical properties, secondary structure, and subcellular localization

The ExPASy database was used for the physical and chemical properties. The PRABI (https://npsa-prabi.ibcp.fr) was used to analyze the secondary structure of VaNF-Y and VvNF-Y protein. Additionally, the WoLF PSORT website (https://wolfpsort.hgc.jp/) was used to predict subcellular localization.

1.4 Phylogenetic tree, conserved domain, motif, and gene structure

The protein sequences of *Arabidopsis*, rice, *V. vinifera*, and *V. amurensis* were used to construct phylogenetic tree using ClustalX and MEGA 7.0 software. The neighbor-joining method was used, and bootstrap replication was 1000. The conservative motif was analyzed using the MEME (https://meme-suite.org/meme/tools/meme). Meanwhile, the motifs of NF-YA, NF-YB, and NF-YC subgroups in *V. vinifera* and *V. amurensis* were identified by MEME, and different conserved motifs of different subgroups were further selected to perform sequence alignment using Geneious Prime software. Additionally, the gene structure analysis was performed using GSDS2.0 (gsds.cbi.pku.edu.cn).

1.5 Cis-acting element, protein interaction, evolutionary selection pressure, synteny

The 2 kb sequences of *NF-YA*, *NF-YB*, and *NF-YC* TFs upstream were screened from the *V. vinifera* and *V. amurensis* genome files using TBtools. The *cis*-acting element components of *NF-YA*, *NF-YB*, and *NF-YC* TFs promoters were identified using the PlantCARE website, and the Simple Biology Sequence Viewer and HeatMap were used to draw the map. Protein interaction analysis was performed using the STRING website, and Cytoscape_3.6.1 software was used to draw the map. The Simple Ka/Ks_Calculator (TBtools) tool was utilized to assess the evolutionary selection pressure placed on the NF-Y TF family using the NG method. The codon usage parameters were determined by analyzing the CDSs of the *NF-Y* genes in *V. vinifera* and *V. amurensis* (Chen et al. 2020). To calculate the divergence time, the Formula T = Ks/2r was employed, with r being considered as 1.5×10^{-8} synonymous substitutions per site per year (Koch et al. 2000). The CDS.fasta files, transcripts.gff (or gff3) files, and genome.fasta files of *V. vinifera*, *V. amurensis*, rice, and *Arabidopsis* were obtained from NCBI and Phytozome v12.1, respectively. These files were utilized to conduct collinearity analysis on *VaNF-Y*, *VvNF-Y*, *AtNF-Y*, and

OsNF-Y TFs. The resulting collinearity was visualized in a circular format using TBtools. Additionally, TBtools were was also used to compare synteny and generate karyotype diagrams between grape and two representative species, namely rice and *Arabidopsis*.

1.6 RNA extraction and RT-qPCR analysis

The *VvNF-Y* and *VaNF-Y* primer sequences were designed and obtained from Biotech Engineering (Shanghai, China) Co, Ltd. via an online synthesis service (Table S1). The Light Cycler® 96 Real-Time PCR System (Roche, Switzerland) was used for the RT-qPCR, with *Vvactin1* serving as the control gene. For RNA extraction, the Omega E.Z.N.A. @plant RNA Kit (Cat: R6827-01, Omega BIO-TEK, Beijing, China) was used to extract RNA from the leaves of 'Pinot Noir' and 'Zuoyouhong' grape plantlets seedings. The Evo M-MLV RT-PCR Kit with gDNA Clean for qPCR (Cat: AG11711, Accurate Biology, Changsha, China) was used to synthesize cDNA for RT-qPCR. The experimental setup included a reaction system with a combined volume of 20 μL, including 10 μL 2×SYBR, 2 μL cDNA, 2 μL upstream and downstream primers, and 6 μL ddH_2O.

1.7 Cloning, vector construction, and transformation of VaNF-YA6 TFs

The CE Design software was used to design forward and revise primers for *VaNF-YA6* (Table S1). The cDNA of 'Zuoyouhong' grape leaves were used to amplified the CDS sequence of *VaNF-YA6* using Green Taq Mix (Cat: P131-AA, Vazyme, Jiangsu, China). Then, the purified fragment was inserted into the *pCAMBIA1300-EGFP* vector by BamH digestion using the ClonExpress®II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). *Agrobacterium* GV3101 carrying *pCAMBIA1300-VaNF-YA6-EGFP* constructs were genetically infected into *Arabidopsis*. The T1 generation seeds were seeded on 1/2 MS and 20 $g{\cdot}L^{-1}$ sucrose medium supplemented with 50 $mg{\cdot}L^{-1}$ kanamycin. The transgenic *Arabidopsis* lines are identified using the Super Light Speed Mix (MF848) reagent kit.

1.8 Subcellular localization of N. benthamiana leaves

Agrobacterium GV3101 carried 35S::VaNF-YA6-EGFP and the control 35S::EGFP protein fusion constructs were genetically introduced into *N. benthamiana* leaves. The fused green fluorescent protein was observed by using a laser scanning confocal microscope (Olympus FV1000 viewer) under low light for 18–24 h. Additionally, 4',6-diamidino-2-phenylindole (DAPI) staining solution is used for nuclear location staining.

1.9 Salt and drought treatment in transgenic Arabidopsis

Wild type (WT) and transgenic *Arabidopsis* were grown on matrix : vermiculite (3:1) medium to the T3 generation materials. For salt stress treatment, the WT and transgenic *Arabidopsis* were subjected to salt treatment for 15 d, the 100 mL solution contained 300 $mmol{\cdot}L^{-1}$ NaCl in per pot. For drought stress treatment, the experimental materials were subjected to natural drought for 15 d. Subsequently, the *Arabidopsis* leaves were collected and quickly frozen at -80 ℃.

1.10 Determination of relative electrical conductivity, malondialdehyde (MDA), proline content, and antioxidant enzyme activity

To study the response of transgenic and WT *Arabidopsis* plants expressing *VaNF-YA6*, they were subjected to NaCl and drought stress conditions. Subsequently, 0.1 g samples were taken to measure the leaves of proline, MDA, and H_2O_2, and the activity of antioxidant enzymes. For determination, Keming assay kits (Catalog nos. MDA-1-Y, H_2O_2-1-Y, POD-1-Y, SOD-1-Y, and CAT-1-Y) were used for the determination. Each treatment was performed in triplicate to ensure reliable results.

1.11 Data processing and statistical analysis

The expression data of *NF-Y* TFs were statistically analyzed using SPSS 22.0. Duncan's test was used to assess significant differences ($P < 0.01$). Bar graphs were generated using GraphPad Prism 5.0. Each treatment was replicated three times to ensure biological reproducibility.

Results

2.1 Physical and chemical properties analysis of NF-Y TFs family

A total of 8 *NF-YA*, 13 *NF-YB*, and 5 *NF-YC* TFs were identified in the *V. amurensis* genome. The longest protein sequence was observed in VaNF-YB9, and the shortest was found in VaNF-YB1. The number of amino acid residues ranged from 104 to 597 aa. The isoelectric point (pI) ranged from 4.60 to 10.13, and the molecular weight ranged from 11.22 to 69.52 KDa. VaNF-YB4 protein had the lowest instability index of 27.66, whereas VaNF-YC5 had the highest instability index of 74.56. The aliphatic index ranged from 51.35 to 87.11, and all NF-Y proteins were hydrophobic proteins.

In the *V. vinifera*, there were the 6 *NF-YA*, 16 *NF-YB*, and 5 *NF-YC* TFs in the NF-Y TF family. The amino acid residue numbers of all NF-Y proteins ranged from 116 to 345 aa. The VvNF-YA4 had the longest protein sequence, and the VvNF-YB9 was the shortest. The pI ranged from 4.62 to 9.76, and the molecular weight ranged from 12.75 to 37.70 KDa. VvNF-YB2 had the lowest instability index of 21.27, and VvNF-YA4 had the highest instability index of 75.43. The aliphatic index ranged from 43.62 to 89.05, and all NF-Y proteins were hydrophobic proteins (Table 1).

Table 1
Physicochemical properties analysis of NF-Y transcription factor members in *Vitis vinifera* and *Vitis amurensis*.

Species name	Gene	Gene accession No.	Amino acid/aa	Molecular weight/KDa	pI	Instability index	Aliphatic index	Hydropathicity
Vitis vinifera	*VvNF-YA1*	*XM_002282742.4*	345	37.7	6.65	70.13	43.62	-1.15
	VvNF-YA2	*XM_002263757.3*	328	36.29	9.18	37.42	61.28	-0.66
	VvNF-YA3	*XM_002274422.3*	226	24.72	9.19	56.89	63.5	-0.66
	VvNF-YA4	*XM_010657434.2*	208	22.65	7.17	75.43	59.71	-0.85
	VvNF-YA5	*XM_002278405.4*	182	20.61	9.76	58.04	62.20	-1.12
	VvNF-YA6	*XM_002278813.2*	234	25.98	9.06	51.00	57.99	-0.92
	VvNF-YB1	*XM_002269460.3*	152	17.26	5.78	26.44	68.68	-0.74
	VvNF-YB2	*XM_010658207.1*	164	18.28	8.86	21.27	78.48	-0.51
	VvNF-YB3	*XM_019219931.1*	197	22.45	4.71	29.91	71.32	-0.53
	VvNF-YB4	*XM_002275446.4*	203	22.7	6.25	55.13	79.31	-0.64
	VvNF-YB5	*XM_010653744.2*	186	21.11	6.45	43.52	65.59	-0.86
	VvNF-YB6	*XM_002276264.3*	208	22.92	7.69	36.02	57.26	-0.72
	VvNF-YB7	*XM_019221403.1*	207	21.91	6.31	27.66	51.35	-0.74
	VvNF-YB8	*XM_002278680.4*	176	19.17	6.42	54.64	59.43	-0.78
	VvNF-YB9	*XM_019225075.1*	132	15.01	5.66	35.57	66.59	-0.86
	VvNF-YB10	*XM_002272151.4*	155	17.34	4.62	48.20	80.00	-0.47
	VvNF-YB11	*XM_003634712.3*	210	22.28	6.44	25.33	48.90	-0.70
	VvNF-YB12	*XM_002265846.3*	170	19.22	6.30	56.75	73.53	-0.59
	VvNF-YB13	*XM_002268446.3*	215	23.87	5.48	57.50	61.81	-0.76
	VvNF-YB14	*XM_003635455.3*	211	23.71	5.91	46.06	61.14	-0.58
	VvNF-YB15	*XM_022903178.1*	215	21.92	6.52	46.94	46.94	-0.65

Species name	Gene	Gene accession No.	Amino acid/aa	Molecular weight/KDa	pI	Instability index	Aliphatic index	Hydropathicity
	VvNF-YB16	*XM_024182139.1*	133	14.56	6.83	43.46	63.91	-0.77
	VvNF-YC1	*XM_002269323.3*	116	12.75	9.46	54.28	89.05	-0.17
	VvNF-YC2	*XM_002280705.4*	273	30.81	8.76	61.16	76.89	-0.53
	VvNF-YC3	*XM_003633662.3*	221	25.04	6.86	59.75	83.94	-0.26
	VvNF-YC4	*XM_002284005.4*	211	23.56	5.06	64.47	72.13	-0.55
	VvNF-YC5	*XM_002262845.4*	182	20.51	5.22	69.07	78.79	-0.36
Vitis amurensis	*VaNF-YA1*	*VAG0107961.1*	218	24.51	7.87	61.81	52.89	-1.02
	VaNF-YA2	*VAG0110210.1*	449	50.19	8.79	52.90	67.57	-0.75
	VaNF-YA3	*VAG0111419.1*	279	31.10	9.39	40.03	62.90	-0.71
	VaNF-YA4	*VAG0111756.1*	560	62.69	6.48	54.77	56.45	-0.82
	VaNF-YA5	*VAG0113942.1*	245	26.90	6.75	74.56	67.84	-0.77
	VaNF-YA6	*VAG0115477.1*	197	21.39	9.69	74.11	57.46	-0.7
	VaNF-YA7	*VAG0117821.1*	376	43.05	9.88	43.81	73.64	-0.57
	VaNF-YA8	*VAG0117824.1*	142	15.71	10.13	37.14	63.87	-0.89
	VaNF-YB1	*VAG0100164.1*	103	11.22	7.68	29.98	80.58	-0.30
	VaNF-YB2	*VAG0107862.1*	133	14.55	6.83	43.46	63.91	-0.77
	VaNF-YB3	*VAG0108441.1*	191	21.64	6.75	44.02	63.87	-0.9
	VaNF-YB4	*VAG0109896.1*	231	25.49	8.27	41.03	62.55	-0.64
	VaNF-YB5	*VAG0113259.1*	207	21.91	6.31	27.66	51.35	-0.74
	VaNF-YB6	*VAG0113262.1*	211	23.70	5.91	47.38	61.1	-0.58
	VaNF-YB7	*VAG0118473.1*	178	19.41	6.09	54.14	60.96	-0.77
	VaNF-YB8	*VAG0127024.1*	215	23.87	5.75	56.24	64.09	-0.71

Species name	Gene	Gene accession No.	Amino acid/aa	Molecular weight/KDa	pI	Instability index	Aliphatic index	Hydropathicity
	VaNF-YB9	*VAG0127025.1*	592	66.33	9.18	45.34	87.11	-0.3
	VaNF-YB10	*VAG0127036.1*	327	35.12	8.69	34.67	66.02	-0.36
	VaNF-YB11	*VAG0129559.1*	175	18.94	5.40	34.05	77.54	-0.24
	VaNF-YB12	*VAG0130297.1*	135	15.21	4.60	52.02	82.44	-0.45
	VaNF-YB13	*VAG0131025.1*	597	69.52	7.74	46.98	80.54	-0.49
	VaNF-YC1	*VAG0105713.1*	281	32.23	9.51	52.56	86.41	-0.36
	VaNF-YC2	*VAG0123197.1*	181	19.97	4.93	52.00	77.07	-0.36
	VaNF-YC3	*VAG0126726.1*	219	24.01	5.08	67.84	76.62	-0.33
	VaNF-YC4	*VAG0129625.1*	363	40.12	8.95	49.69	76.17	-0.44
	VaNF-YC5	*VAG0131857.1*	233	26.13	6.38	56.34	69.96	-0.55

2.2 Phylogenetic tree analysis

The construction of the phylogenetic tree was constructed using the amino acid sequences of the *NF-Y* TFs from *Arabidopsis*, rice, *V. vinifera* and *V. amurensis* (Fig. 1). The analysis results of the phylogenetic tree suggested that the NF-Y TF family was divided into three distinct subgroups. The *NF-YA* TFs were mainly clustered in clade A, comprising a total of 34 members (10 from *Arabidopsis*, 10 from rice, 8 from *V. vinifera* and 6 from *V. amurensis*). Clade B consists mainly of 51 members of *NF-YB* TFs members (11 from *Arabidopsis*, 11 from rice, 13 from *V. vinifera* and 16 from *V. amurensis*). Clade C had only 29 members of the *NF-YC* TFs members (10 from *Arabidopsis*, 9 from rice, 5 from *V. vinifera* and 5 from *V. amurensis*).

2.3 Subcellular localization and protein secondary structure analysis

The predicted results of the protein secondary structure showed that all NF-Y proteins in *V. vinifera* and *V. amurensis* were predominantly alpha-helix and random coil. However, there were also clear differences between the different subgroups. Specifically, the alpha-helix content in VvNF-YA proteins ranged from 13.04–29.12% in *V. vinifera*, while in *V. amurensis* it ranged from 16.90–29.36%. The random coil content in VvNF-YA proteins ranges from 54.95–77.68% in *V. vinifera*, while in *V. amurensis* it ranges from 52.75–64.79%. For the VvNF-YB and VvNF-YC proteins, there were clear differences between *V. vinifera* and *V. amurensis* (Table 2).

Table 2
Secondary structure and subcellular localization prediction of NF-Y proteins in *Vitis vinifera* and *Vitis amurensis*.

Species name	Transcription factors	Alpha helix/%	Extended strand/%	Beta turn/%	Random coil/%	Subcellular location
Vitis vinifera	*VvNF-YA1*	13.04	7.25	2.03	77.68	nucl: 13, chlo: 1
	VvNF-YA2	15.85	13.11	3.35	67.68	nucl: 14
	VvNF-YA3	17.26	8.85	7.08	66.81	nucl: 14
	VvNF-YA4	18.27	7.69	2.40	71.63	nucl: 13
	VvNF-YA5	29.12	9.34	6.59	54.95	nucl: 13, chlo: 1
	VvNF-YA6	17.95	12.39	2.56	67.09	nucl: 13, chlo: 1
	VvNF-YB1	63.16	4.61	4.61	27.63	cyto: 4, mito: 4
	VvNF-YB2	48.17	7.32	3.05	41.46	chlo: 2, nucl: 2,
	VvNF-YB3	63.96	5.58	5.58	24.87	nucl: 13,
	VvNF-YB4	47.78	10.84	4.39	36.45	nucl: 4, chlo: 2,
	VvNF-YB5	46.77	5.38	4.84	43.01	nucl: 13,
	VvNF-YB6	35.58	11.54	7.21	45.67	nucl: 12,
	VvNF-YB7	40.10	11.11	7.25	41.55	nucl: 14
	VvNF-YB8	41.48	5.11	6.25	47.16	nucl: 14
	VvNF-YB9	52.27	6.82	5.30	35.61	nucl: 5, chlo: 1,
	VvNF-YB10	62.58	1.29	1.94	34.19	chlo: 3, nucl: 1.5,
	VvNF-YB11	63.23	0.65	1.94	34.19	nucl: 14
	VvNF-YB12	35.71	3.81	4.76	55.71	chlo: 4, nucl: 3.5,
	VvNF-YB13	39.07	8.84	7.44	44.65	mito: 7, nucl: 3
	VvNF-YB14	38.39	2.84	0.95	57.82	cyto: 3, nucl: 2
	VvNF-YB15	36.74	8.37	7.44	47.44	nucl: 14
	VvNF-YB16	51.13	1.50	6.77	40.60	nucl: 14
	VvNF-YC1	50.00	9.48	3.45	37.07	chlo: 8
	VvNF-YC2	42.49	14.29	8.42	34.80	chlo: 5, nucl: 2.5,
	VvNF-YC3	51.49	21.64	6.72	20.15	plas: 5.5
	VvNF-YC4	44.55	7.58	5.69	42.18	nucl: 6,
	VvNF-YC5	37.91	4.95	1.65	55.49	nucl: 7, chlo: 1
Vitis amurensis	*VaNF-YA1*	29.36	9.63	8.26	52.75	nucl: 12,
	VaNF-YA2	22.94	11.58	4.01	61.47	nucl: 12.5,
	VaNF-YA3	20.79	13.26	5.02	60.93	nucl: 8, chlo: 2,

Note: nucl: nucleus, chlo: chloroplast, cyto: cytoplasm, mito: mitochondria, vacu: vacuole, plas: plasma membrane, extr: extracellular

Species name	Transcription factors	Alpha helix/%	Extended strand/%	Beta turn/%	Random coil/%	Subcellular location
	VaNF-YA4	20.36	12.86	6.61	60.18	nucl: 13,
	VaNF-YA5	25.31	13.06	3.27	58.37	extr: 6, vacu: 6
	VaNF-YA6	17.83	13.95	5.04	63.18	nucl: 12
	VaNF-YA7	18.35	19.41	5.85	56.38	nucl: 8
	VaNF-YA8	16.90	13.38	4.93	64.79	nucl: 8
	VaNF-YB1	55.34	8.74	4.85	31.07	mito: 7
	VaNF-YB2	51.13	1.50	6.77	40.60	nucl: 14
	VaNF-YB3	40.10	11.11	7.25	41.55	nucl: 14
	VaNF-YB4	40.69	12.12	4.76	42.42	nucl: 13
	VaNF-YB5	39.27	8.38	8.38	43.98	nucl: 14
	VaNF-YB6	38.39	2.84	3.32	55.45	nucl: 4,
	VaNF-YB7	44.38	5.06	5.06	45.51	nucl: 14
	VaNF-YB8	45.12	6.05	6.98	41.86	mito: 7
	VaNF-YB9	31.08	16.55	8.45	43.92	mito: 7.5
	VaNF-YB10	36.39	7.34	5.81	50.46	nucl: 13, chlo: 1
	VaNF-YB11	45.71	13.71	8.00	32.57	cyto: 10
	VaNF-YB12	71.11	0	2.22	26.67	chlo: 7, nucl: 2.5,
	VaNF-YB13	44.56	12.06	3.35	40.03	chlo: 5, nucl: 3,
	VaNF-YC1	43.77	12.10	6.05	38.08	mito: 3.5, nucl: 3,
	VaNF-YC2	39.23	10.50	6.63	43.65	nucl: 5, chlo: 1
	VaNF-YC3	30.14	12.33	2.74	54.79	cyto: 10, nucl: 4
	VaNF-YC4	57.58	10.74	5.79	25.90	chlo: 7
	VaNF-YC5	35.19	14.16	3.86	46.78	nucl: 9

Note: nucl: nucleus, chlo: chloroplast, cyto: cytoplasm, mito: mitochondria, vacu: vacuole, plas: plasma membrane, extr: extracellular

The subcellular localization analysis of the NF-Y proteins belonging to *V. vinifera* and *V. amurensis* showed that the majority of these family members were located in the nucleus, with a few proteins also expressed in chloroplasts, cytoplasm, mitochondria and plasma membrane in *V. vinifera*. In *V. amurensis*, compared to *V. vinifera*, all *NF-Y* TFs were also mainly expressed in the nucleus. In addition, VaNF-YA3, VaNF-YB1, VaNF-YB8, VaNF-YB9, VaNF-YB10, VaNF-YB11, VaNF-YB12, VaNF-YB13, VaNF-YC1, VaNF-YC2, VaNF-YC3, VaNF-YC4 were expressed in chloroplasts, cytoplasm, mitochondria and vacuole. Interestingly, TFs are not observed to be expressed on the plasma membrane, whereas VaNF-YA5 proteins show extracellular expression (Table 2).

2.4 Multi sequence alignment analysis

The NF-Y protein sequences of *V. vinifera* and *V. amurensis* were used to perform a multiple sequence alignment analysis. All three subfamilies of NF-Y protein sequences contained DNA binding domains. In addition, the NF-YA subunit contained the NF-YB and NF-YC interaction domains, while the NF-YB subunit had two distinct structural domains, namely NF-YA interaction and NF-YC interaction. Similarly, NF-YC also had two structural domains, the NF-YA interaction domain and the NF-YB interaction domain (Figure S1).

2.5 Motif, conserved domain, and gene structure analysis

Five motifs were identified in *V. vinifera* and *V. amurensis*, the subgroups NF-YA, NF-YB, NF-YC had similar motifs. The NF-YA subgroup mainly includes motif 1, motif 2, and motif 4, the NF-YB subgroup mainly includes motif 1 and motif 5, the NF-YC subgroup only mainly includes motif 3. All conserved motif amino acid residues range from 21 to 50 aa (Fig. 2A and 2B). The conserved domains analysis results showed that the NF-YA, NF-YB, and NF-YC subgroup proteins mainly included CBFB_NF-YA, CBFD_NF-YB_HMF, and HAP5 superfamily, respectively. Additionally, some TFs also had SIRT7, SIR2, Remorin_C and others (Fig. 2C).

Analysis of gene structures showed that *VaNF-YB9* had the longest gene structure, and the same subgroups had similar structures. Interestingly, in *V. vinifera*, the length for exons of the NF-YA subgroup ranged from 627 bp to 1053 bp. The length for exons of the NF-YB subgroup ranged from 417 bp to 648 bp. The length for exons of the NF-YC subgroup ranged from 350 bp to 810 bp. In *V. amurensis*, the length for exons of the NF-YA subgroup ranged from 429 bp to 1683 bp. The length for exons of the NF-YB subgroup ranged from 312 bp to 984 bp. The length for exons of the NF-YC subgroup ranged from 546 bp to 1092 bp. The number of exons in *V. vinifera* ranges from 1 to 7, while it ranges from 1 to 14 in *V. amurensis* (Fig. 2D and Fig. 3A and 3B).

In addition, the five motifs of the NF-YA, NF-YB, NF-YC subgroups were further identified and compared between *V. vinifera* and *V. amurensis* (Figure S2-S7). The results of the analysis show a considerable discrepancy in the protein sequence of the NF-YA subgroup, especially in the regions of the N-terminus and C-terminus. This discrepancy mainly involved the loss or gain of protein sequences. For example, the NF-YA protein sequence of 'NTKKLDWEFWGCCDDCEKWFGGCC' was lost in the *V. vinifera* compared to *V. amurensis*, whereas the sequence 'SSVYSQPWWGHSIVCVA' was gained. For the NF-YB subgroup, the protein sequences showed higher conservation compared to the NF-YA and NF-YC subgroups. In the context of C-termination, the VaNF-YB protein sequences differed from the VvNF-YB protein sequences by only three amino acids (ANQ). However, in the NF-YC subgroup, the *V. vinifera* and *V. amurensis* protein sequences differed slightly from the NF-YA protein sequences (Fig. 3C-3E).

2.6 Collinearity analysis of NF-Y TF family in grapes

To gain insight into the chromosomal position and duplication of the *VaNF-Y* and *VvNF-Y* TFs, an analysis was performed to identify the synteny relationships of the *NF-Y* TFs. The results of the chromosomal position analysis showed that the *VaNF-Y* TFs had a predominant distribution on fourteen chromosomes, namely 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17, 19. Within these chromosomes, chromosome 19 harbored the highest number of *VvNF-Y* TFs, specifically *VvNF-YB11*, *VvNF-Y12*, *VvNF-YB13* and *VvNF-YC5* (Fig. 4A). However, the *VaNF-Y* TFs were predominantly scattered over eleven chromosomes. Among these chromosomes, 19 chromosomes contained four TFs, namely *VaNF-YB8*, *VaNF-Y9*, *VaNF-YB10* and *VaNF-YC3*. In addition, chromosomes 10 and 13 contained three *VaNF-Y* TFs. Gene pair synteny analysis revealed that there were three gene pairs of collinear TFs for the *V. vinifera VvNF-Y* TFs, including *VaNF-YA1/VaNF-YA6*, *VaNF-YA3/VaNF-YA5*, and *VaNF-YB7/VaNF-YB16*. Meanwhile, four gene pairs were identified in the *VaNF-Y* TFs, namely *VaNF-YA1/VaNF-YA7*, *VaNF-YA4/VaNF-YA6*, *VaNF-YB2/VaNF-YB7* and *VaNF-YB5/VaNF-YB10* (Fig. 4B).

An analysis was also carried out to determine the genome-wide synteny among *V. vinifera*, *V. amurensis*, *Arabidopsis*, and rice. In this study, it was observed that there were 30 gene pairs between *V. vinifera* and *V. amurensis*. These gene pairs were

identified as Chr05, Chr06, Chr07, Chr08, Chr09, Chr10, Chr11, Chr13, Chr17 and Chr19 in *V. amurensis* and 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17 and 19 in *V. vinifera* in karyotype plots (Fig. 4C). The synteny relationship was further analyzed between the two grape species and two representative species (*Arabidopsis* and rice), and it was found that *V. vinifera* shared 10 and 11 *NF-Y* gene pairs with *Arabidopsis* and rice, respectively. In addition, *V. amurensis* had 10 and 7 gene pairs with *Arabidopsis* and rice, respectively (Fig. 4D-4E).

2.7 Protein interaction analysis of VaNF-Y and VvNF-Y proteins

The protein interaction analysis of NF-Y proteins was performed for NF-Y proteins in *V. vinifera* and *V. amurensis* (Fig. 2–7). The protein interaction analysis results showed that there were obvious protein interaction relationships between the NF-YA, NF-YB and NF-YC subgroups. However, in *V. amurensis*, VaNF-YA8, VaNF-YB9, VaNF-YB12 and VaNF-Y13 proteins had no interaction relationship with other VaNF-Y proteins. Similarly, VvNF-YB16 had no interaction relationship with other VvNF-Y proteins in *V. vinifera* (Fig. 5).

2.8 codon usage parameters analysis

Furthermore, in both *V. vinifera* and *V. amurensis*, the third base of *NF-Y* TFs showed a higher tendency to use the GC of the synonymous codon compared to others in third codons. The codon adaptation index (CAI) and codon bias index (CBI) values were found to be less than 0.286 and 0.215, respectively, suggesting that the *VaNF-Y* and *VvNF-Y* TFs may use less high expression superior codon and optimal codon in *V. vinifera* and *V. amurensis*. Analysis of the frequency of optimal codon (FOP) parameter showed that all *NF-Y* TFs ranged from 0.314 to 0.551 (Table S2).

Meanwhile, the number of codon usage of *VaNF-Y* and *VvNF-Y* TFs was further analysed. The analysis results showed that the *VaNF-Y* and *VvNF-Y* TFs were similar in codon usage in *V. vinifera* and *V. amurensis*. However, there are significant differences in the codons used for different amino acids. Arg is preferentially encoded by AUG and AGU. Leu is preferentially encoded by UUG, CUU and CUG. Val is preferentially encoded by CUU and GUG, and so on (Fig. 6A and 6B and Figure S8).

In addition, a correlation analysis was performed to examine the relationship between different codon usage parameters, which revealed clear differences between *V. vinifera* and *V. amurensis*. In *V. amurensis*, the CBI and Fop values of *VaNF-Y* TFs showed a significant and positive correlation with C3s, GC and CAI, whereas T3s showed a significant and negative correlation with CBI and Fop (Fig. 6C). In *V. vinifera*, the codon correlation was similar to that of *V. amurensis*, with only an increased difference in the correlation between L_sym, L_aa, Gravy and Aromo parameters (Fig. 6D).

2.9 Cis-acting element analysis

The *VvNF-Y* and *VaNF-Y* TFs upstream analysis of the 2 kb sequences revealed that the majority of four hormones and five stress *cis*-acting elements were identified in the *NF-Y* TFs. Among these, especially anaerobic response elements are found in most of the *NF-Y* TFs. We speculate that this family of TFs responds to stress by increasing antioxidant enzyme activity to improve grapevine resistance. Interestingly, the promoters of *NF-YA* and *NF-YB* subgroups TFs had more MeJA and ABA cis-acting elements in the *V. amurensis*. For example, in the *VaNF-YA2* and *VaNF-YB13* promoters, the highest number of MeJA (10 from *VaNF-YA2* and 8 from *VaNF-YB13*) and ABA (9 from *VaNF-YA2* and 10 from *VaNF-YB13*) responsive elements were identified, as shown in Fig. 7. Thus, these elements may have important roles in defense against hormones, biotic, and abiotic stresses.

2.10 The expression levels of the VvNF-Y and VaNF-Y TFs under NaCl and PEG stresses

The expression levels of *VaNF-Y* and *VvNF-Y* TFs in *V. vinifera* and *V. amurensis* leaves were examined by RT-qPCR under and PEG treatments for 24 h. The same *VaNF-Y* and *VvNF-Y* TFs expression levels in NF-YA, NF-YB, and NF-YC subgroups

were obviously upregulated under NaCl and PEG treatments for 24 h (Fig. 8).

In *V. amurensis*, the expression levels of *VaNF-YA2*, *VaNF-YA6*, *VaNF-YB5*, *VaNF-YB9*, *VaNF-YB11*, *VaNF-YC2*, *VaNF-YC3*, and *VaNF-YC4* in leaves were significantly upregulated after 24 h treatment with NaCl and PEG compared to 0 h. In particular, *VaNF-YB5*, *VaNF-YA6*, *VaNF-YB9*, and *VaNF-YB11* were strongly induced by about 11.64- and 19.70-, 75.70-, and 42.43-fold, 13.55- and 10.54-, 9.18-, and 6.93-fold under NaCl and PEG treatments for 24 h respectively. Meanwhile, the expression levels of *VaNF-YA5*, *VaNF-YB3*, *VaNF-YB4*, *VaNF-YB6* and *VaNF-YC5* also showed significant downregulation under NaCl and PEG stress for 24 h (Fig. 8A-8C).

In addition, the expression levels of *VvNF-YA3*, *VvNF-YA5*, *VvNF-YB3*, *VvNF-YB5*, *VvNF-YB8*, *VvNF-YB14*, *VvNF-YB15*, *VvNF-YB16*, *VvNF-YC2*, and *VvNF-YC5* in 'Pinot Noir' leaves were significantly upregulated after 24 h of NaCl and PEG treatment compared to 0 h. Especially, *VvNF-YA3*, *VvNF-YA5*, *VvNF-YB3*, *VvNF-YB5*, and *VvNF-YC2* were strongly induced under NaCl and PEG treatments for 24 h by approximately 11.33- and 9.85-fold, 10.68- and 12.57-fold, 17.14- and 11.70-fold, 7.88- and 9.07-fold, 16.01- and 13.21-fold, respectively. Meanwhile, the expression levels of *VvNF-YA4*, *VvNF-YA6*, *VvNF-YB1*, *VvNF-YB9*, *VvNF-YB12*, *VvNF-YC1*, and *VvNF-YC4* showed significant downregulation under NaCl and PEG stress for 24 h. Interestingly, the expression level of *VvNF-YB14* showed significant upregulation under PEG treatment, while it was slightly upregulated under salt treatment (Fig. 8D-8F).

2.11 PCR amplification, vector construction, and subcellular localization

The present study involves a comprehensive bioinformatic study of the *NF-Y* TFs, followed by expression analysis under NaCl and PEG stress. RT-qPCR analysis identified *VaNF-YA6* that showed significant up-regulation in response to salt and drought stress. Therefore, primers were designed to amplify *VaNF-YA6* CDS sequences, resulting in successful PCR amplification that reflected the CDS length of *VaNF-YA6* (594 bp) (Figure S9A). Furthermore, the purified fragment was successfully inserted into pCAMBIA2300-EGFP to form the pCAMBIA2300-VaNF-YA6-EGFP fusion vector and subsequently introduced into GV3101 for *Arabidopsis* infection and subcellular localization (Figure S9B and S9C). *A. tumefaciens* was introduced into the dorsal region of tobacco leaves to achieve transient expression. The samples were then observed by confocal microscopy after 24 h. The results showed a predominant presence of green fluorescence in the nucleus, indicating the nuclear localization and functionality of *VaNF-YA6* (Fig. 9A-9B).

2.12 Phenotypic and expression analysis of VaNF-YA6 transgenic Arabidopsis under salt and drought stress

PCR is used to identify positive transgenic *Arabidopsis*. Finally, five positive transgenic lines were successfully identified. Among them, three transgenic lines, OE-3, OE-6, and OE-7, were further selected to verify the stress function (Figure S9D). Experimental drought treatment was performed on WT and transgenic *Arabidopsis* lines. Both overexpressed *Arabidopsis* and WT materials were irrigated with 300 $mmol \cdot L^{-1}$ NaCl, and their phenotypic changes were recorded before and after treatments. After NaCl treatment, the yellowing and curling of leaves were more severe in WT. Overexpression lines showed normal leaf growth except for some yellowing (Fig. 9C). RT-qPCR analysis of transgenic and WT *Arabidopsis* showed that the expression level of *VaNF-YA6* was significantly upregulated by approximately 45-, 47-, and 46-fold higher than in WT *Arabidopsis*, respectively (Fig. 9D).

Under the natural drought treatment, the leaves of WT plants showed more severe wilting, whereas the transgenic plants showed normal growth, except for some yellowing leaves (Fig. 9C). Additionally, RT-qPCR was used to assess the expression level of the *VaNF-YA6* in both WT and transgenic *Arabidopsis* lines. The results showed a significant upregulation of the *VaNF-YA6* after the drought treatment, with expression levels 37-, 35-, and 36-fold higher than WT, respectively (Fig. 9E).

2.13 Determination of relative electrical conductivity, malondialdehyde, H_2O_2, antioxidant enzyme activity and proline contents

Similarly, the proline content in leaves was significantly increased in transgenic *Arabidopsis* compared to WT. Additionally, relative electrical conductivity and MDA content were measured to assess the osmotic stress on cell membranes. The results showed an increase in relative conductivity and MDA content in WT after NaCl and drought stress. Therefore, overexpression of the *VaNF-YA6* in *Arabidopsis* alleviates osmotic stress on cell membranes during drought stress (Fig. 9F-9H).

Hydrogen peroxide (H_2O_2) levels in transgenic *Arabidopsis* and WT leaves were assessed before and after 15 d under NaCl natural drought treatment. The results showed an overall increase in H_2O_2 levels after exposure to NaCl and drought stress. However, the H_2O_2 levels in leaves of *Arabidopsis* overexpressing lines of the *VaNF-YA6* were lower compared to WT, suggesting that the overexpression of the *VaNF-YA6* in *Arabidopsis* reduces the accumulation of reactive oxygen.

Antioxidant enzymes have the ability to convert excess reactive oxygen species and free radicals into less harmful substances, thereby balancing the levels of reactive oxygen species in the body. This is an essential physiological indicator for plants in coping with abiotic stress. The POD activity was measured in *Arabidopsis* and it was found that after treatment with 300 $mmol{\cdot}L^{-1}$ NaCl, the POD, SOD, and CAT activity in transgenic *Arabidopsis* were significantly enhanced compared to WT. The above experimental results indicate that the *VaNF-YA6* enhances the salt tolerance of overexpressed *Arabidopsis* lines by increasing the activity of antioxidant enzymes in transgenic *Arabidopsis* (Fig. 10A, 10C, 10E, 10G).

Under natural drought treatment, the activities of these enzymes were higher in transgenic *Arabidopsis* compared to the WT. The measurement results of proline content in leaves were consistent with the enzyme activities, as both were higher in transgenic *Arabidopsis* lines compared to WT under drought treatment for 15 d. The *VaNF-YA6* in transgenic *Arabidopsis* enhances the resistance to drought stress mainly by increasing the activity of antioxidant enzymes (Fig. 10B, 10D, 10F, 10H).

Discussion

NF-Y TFs are commonly found in plants as a gene family consisting of the three subunits NF-YA/B/C, which adds complexity and variability to their functions (Hackenberg et al. 2012). To date, members of the NF-Y TF family have been identified in the numerous species, including *Arabidopsis* (Bhattacharjee and Hallan 2023), *Oryza sativa* (rice) (Thirumurugan et al. 2008), *Glycine max* (soybean) (Quach et al. 2015), V. *vinifera* (Ren et al. 2016), wild jujuba (Panzade et al. 2022), *Musa nana* (banana) (Yan et al. 2019), *Hordeum vulgare* (barley) (Panahi et al. 2019), *Populus* (poplar) (Liu et al. 2021), potato (Li et al. 2021), *Prunus persica* (peach) (Li et al. 2019), and wheat (Stephenson et al. 2007), and so on. In this study, we also identified 26 (6 from *VvNF-YA*, 16 from *VvNF-YB*, and 5 from *VvNF-YC*) and 27 (8 from *VaNF-YA*, 13 from *VaNF-YB*, and 5 from *VaNF-YC*) NF-Y TFs from *V. vinifera* and *V. amurensis*, respectively, and all the *NF-Y* TFs were divided into three subgroups, NF-YA, NF-YB, and NF-YC. The similar results were found in melon, Chinese cabbage, and Chrysanthemum (Hu, et al. 2023; Jiang, et al. 2023; Li, et al. 2023). The gene structure analysis results showed that the *NF-Y* TFs in the NF-YA, NF-YB, and NF-YC subgroups contained similar numbers of exons and introns. Meanwhile, different subgroups had unique conserved domains, including CBFB_NF-YA from the NF-YA subgroup, CBFD_NF-YB_HMF from the NF-YB subgroup, and the HAP5 superfamily from the NF-YC subgroup. Similar functional domains were also discovered in Chinese cabbage (Jiang et al. 2023). Although collinearity relationship was found to contain 36 collinearity genes in Chinese cabbage, whereas only four and three pairs of collinearity genes pairs were found among the three subgroups in *V. amurensis* and *V. vinifera*, respectively (Jiang et al. 2023). A similar result also was found in Chrysanthemum. Additionally, five motifs were identified in *V. amurensis* and *V. vinifera*, there were motif 1, motif 2, and motif 4 in the NF-YA subgroup,

motif 1 and motif 5 in the NF-YB subgroup, and motif 3 in NF-YC subgroup (Hu et al. 2023). Interestingly, five motifs from *V. amurensis* and *V. vinifera* were further analyzed. We found that *NF-YB* TFs in grape showed higher conservation than other subgroups. In addition, the amino acid sequences of NF-YA and NF-YC sequences showed varying degrees of loss and gain in both species. Thus, we speculated that these sequences may be closely related to the functions performed by grapevine.

Previous studies have shown that *NF-YB* and *NF-YC* assemble as dimers in the cytoplasm and then translocate to the nucleus, whereas their main function is to form a fully functional heterotrimeric complex with NF-YA when NF-YB and NF-YC enter the nucleus (Frontini et al. 2004). In this study, subcellular localization prediction analysis revealed that most of the *NF-YA* TFs are mainly located in the nucleus, whereas *NF-YB* and *NF-YC* TFs are located in the nucleus and other organelles. Furthermore, subcellular localization analysis of VaNF-YA6 in tobacco was also located in the nucleus. *Cis*-acting element analysis of *VaNF-Y* and *VvNF-Y* TFs revealed most of the elements related to low temperature, drought, anaerobic induction, defense and stress, ABA, IAA, GA3 and MeJA, indicating their role in stress resistance. Similarly, most of the elements related to osmotic stress and hormones were found in chrysanthemum (Hu et al. 2023). Moreover, anaerobic response elements are found in most of the *NF-Y* TFs. We speculate that *NF-Y* TFs responds to stress by increasing antioxidant enzyme activity to improve grapevine resistance. Meanwhile, the promoters of *NF-YA* and *NF-YB* subgroups TFs had more MeJA and ABA *cis*-acting elements in the *V. amurensis* compared to *V. vinifera*.

The *NF-Y* TF family plays various roles in seed germination, embryo growth and development, flowering, and stress response to elements, hormones, and high salt concentrations (Yang et al. 2005). In this study, RT-qPCR analysis showed that many *VaNF-Y* and *VvNF-Y* TFs were up-regulated under drought and salt treatments at 24 h in *V. amurensis* and *V. vinifera*, respectively. In particular, *VaNF-YA6*, *VaNF-YB5*, *VaNF-YC3*, *VvNF-YA5*, *VvNF-YB14*, *VvNF-YC2*, etc. showed significant upregulation. In *Arabidopsis*, the *AtNF-YA5* expression was increased, affecting plant water uptake and stomata, and inducing drought-related gene expression through the ABA pathway (Hackenberg et al. 2012; Warpeha et al. 2007). In soybean, overexpression of *GmNF-YA5* and *GmNF-YCa* also increased drought resistance through the ABA pathway (Hackenberg et al. 2012; Ma et al. 2020; Warpeha et al. 2007).

In the face of unfavorable conditions, plants exhibit a number of physiological and biochemical responses aimed at protecting themselves from the detrimental effects of adversity. Key compounds such as MDA, proline, H_2O_2, and antioxidant enzymes play a pivotal role in enhancing plant resilience to stress-induced damage (Kusvuran et al. 2016; Seckin et al. 2010). To gain a deeper understanding of the functions of the *VaNF-YA6* in grape, it was cloned and transformed into *Arabidopsis*. Subsequent experiments showed that WT *Arabidopsis* exhibited severe leaf yellowing and curling under high concentrations of salt and drought stress compared to the transgenic *Arabidopsis* lines. Meanwhile, the expression levels of the transgenic lines showed obvious upregulation under salt and drought stresses for 15 d compared to WT. Similarly, overexpression of *PdNF-YB7* of polar showed increased root length and leaf area in transgenic *Arabidopsis* by improving water utilization and enhancing drought resistance (Han et al. 2013). The *AsNF-YC8* was found to increase salt and drought tolerance in transgenic lines compared to WT tobacco (Nelson et al. 2007). Meanwhile, *Cdt-NF-YC1* significantly improved the salt and alkali tolerance of rice (Chen et al. 2015). Additionally, the determination of H_2O_2, REC, and MDA contents demonstrated that the accumulation of reactive oxygen species in leaves was lower in transgenic *Arabidopsis* lines compared to WT after salt and drought treatments. Therefore, we assumed that the overexpression of the *VaNF-YA6* in *Arabidopsis* can reduce cell membrane damage under drought stress. In addition, the determination of POD, SOD, CAT activities, and proline content revealed that transgenic *Arabidopsis* lines exhibited higher levels of these enzyme activities and proline content than WT. Therefore, overexpression of *VaNF-YA6* appeared to enhance salt and drought tolerance in transgenic *Arabidopsis*. *SiNF-YA1* in millet was able to activate stress-related genes and enzyme activities, chlorophyll content, and relative water content, thereby promoting drought and salt stress resistance (Feng et al. 2015).

In this study, a bioinformatic comparative analysis was performed on the *NF-Y* TF family between *V. vinifera* and *V. amurensis*. A total of 27 and 26 *NF-Y* TF members were identified from *V. vinifera* and *V. amurensis*, respectively, and divided into 3 subfamilies. Interestingly, the conserved five motif analysis showed that NF-YB protein sequences had higher

conservation, whereas the amino acid sequences of NF-YA and NF-YC sequences showed varying degrees of loss and gain in both species. Thus, these sequences may be closely related to the functions performed by grapevine. Under NaCl and PEG treatment, the expression levels of *VaNF-YA6*, *VaNF-YB5*, *VaNF-YC3*, *VvNF-YA5*, *VvNF-YB14*, and *VvNF-YC2* members were significantly upregulated. Meanwhile, the subcellular localization of *VaNF-YA6-GFP* in tobacco leaves was mainly located in the nucleus, and overexpression of *VaNF-YA6* improved the tolerance to salt and drought tolerance in *Arabidopsis* by improving enzyme activities and other protective substance contents.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This work was supported by the Key Projects of National Natural Science Foundation in Gansu Province (22JR5RA831), the 2022 Modern Silk Road Cold and Drought Agricultural Science and Technology Support Project (GSLK-2022-4), and Excellent Doctoral Project of Gansu Province (22JR5RA845).

Author Contribution Statement

Shixiong Lu designed the experiments, coordinated, and organized the whole research activities. Xueting Zhou, Xu Huang, Baozhen Zeng, Huimin Gou, Zonghuan Ma, and Shixiong Lu participated in most of the experiments and data collection. Xueting Zhou and Xu Huang provided technical assistance to Shixiong Lu. Shixiong Lu wrote the manuscript with contributions from all the authors. Shixiong Lu and Xu Huang drew all the figures and tables. Baihong Chen and Juan Mao revised the manuscript. All authors read, reviewed, and approved the final manuscript.

Acknowledgments

The authors were thankful to the Key Projects of National Natural Science Foundation in Gansu Province (22JR5RA831), the 2022 Modern Silk Road Cold and Drought Agricultural Science and Technology Support Project (GSLK-2022-4), and Excellent Doctoral Project of Gansu Province (22JR5RA845). The authors would also like to thank all authors for their help and the funding agencies for financing the experimental cost for this work.

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